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INTRODUCTION:

In this Synergy award, we proposed to bring compounds identified with a novel screening method to standard mouse breast cancer assays. We developed Csk/Src Drosophila models to explore specific aspects of overgrowth and metastasis. Both Src and the Csk paralog Chk have been implicated in breast cancer metastasis. We propose to test the hypothesis that drugs identified in our novel Drosophila wing model of tumor (invasive proliferation) and metastasis— targeting the effects of activated Src— will show efficacy in a mouse model of breast cancer and metastasis. The overall goal of this proposal is to validate the findings from a *Drosophila* metastasis model in murine and human breast cancers. Specifically, we will examine the interactions of epithelial tumor cells with bordering non-malignant epithelial cells, and whether these interactions alter the metastatic potential of cells at the tumor boundary. Several critical signaling pathways specific to this interaction have been identified in a *Drosophila* whole animal genetic screen. We propose to validate these pathways in mouse and human breast cancers, and to extend the *Drosophila* search for new factors. The long term goals of this proposal will be to identify critical targets involved in tumor progression and breast cancer metastasis using the power of forward genetics in *Drosophila*, and to develop novel murine breast cancer models of metastasis that can be used to screen new genes and therapeutics targeted to breast cancer metastasis (**Figure 1**).

BODY:

Our efforts were strongly successful, and we now have two joint manuscripts under review that presents our work as a new approach to breast cancer therapeutics. We have also written a clinical trial using the **NCI/CTEP** hedgehog inhibitor, GDC-0449, in patients with locally advanced breast cancer. (See **Appendix 1** for CTEP/NCI letter of intent). We identified hedgehog antagonists as powerful suppressors of metastases in both the fly cancer models and murine breast cancer models and we now develop a clinical trial in patients with breast cancer.

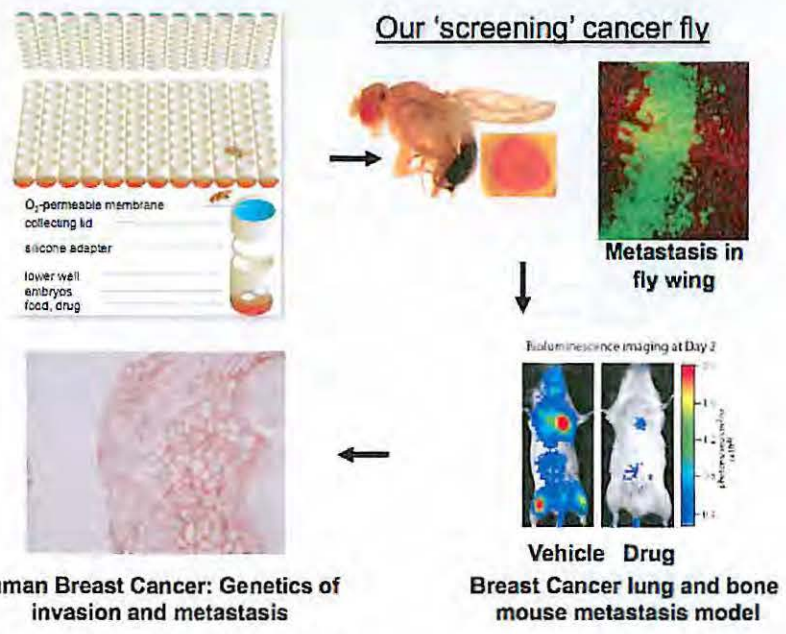


Figure 1. Overview of approach

We have also submitted a **Breast Cancer SPORE proposal** entitled "Targeting Hedgeghog signaling to prevent breast cancer metastases".

Manuscripts under review:

1) **Whole Animal Approach to Cancer Therapeutics Screening**. Hirabayashi, S, Marcos V., Graves, J, Fink, J., Heller, E., Weilbaecher, K., and Cagan, R.. Under review at *Nature Methods*.

Abstract: We present a novel method of whole animal compound screening using Drosophila melanogaster. Utilizing a mutligenic Ras/Csk tumor model, we calibrated enhancement of tumor growth and metastasis by altering dietary sugar. We then screened chemical libraries to identify several compounds that successfully suppressed tumor growth and metastasis. Our approach provides a template for large-scale whole animal screening that relies on robotics as well as rescue from lethality (**Figure 1**).

2) **A Role for the Epithelial Microenvironment at Tumor Boundaries: Evidence from Drosophila and Human Squamous Cell Carcinomas.** Marcos Vidal[1,2], Lorena Salavaggione[3] , Lourdes Ylagan4, Mark Wilkins, Mark Watson3, Katherine Weilbaecher5 and Ross Cagan. Under review at *Am J Pathology*.

Abstract: Recent work in cancer has shown an increasing appreciation for the importance of the tumor's environment and the overlying stroma. Less emphasis has been placed on the importance of local communication between transformed cells and their neighbors within the epithelium at tumor boundaries. We previously reported a *Drosophila* tumor model that highlighted the importance of local interactions within the epithelial microenvironment: genotypically src (*Csk*-) transformed cells were influenced by their immediate, normal neighbors. The result of this interaction was a consistent change in the 'border cells' at the tumor's edge including de-localized p120-catenin and E-cadherin as well as invasive migration through the basal lamina. Here we show that the invasive properties of the boundary tumor cells depend on up-regulation of *Drosophila* Matrix Metalloproteinase-1 (MMP1) as assessed by promoter activity, protein levels, *in situ* enzymatic activity, and tests of genetic modifier activity. Utilizing epitope-specific antibodies, we identified similar changes in 'boundary cells' within histologic sections of human Squamous Cell Carcinomas (SCC) as observed in *Drosophila* epithelial tumors. Both E-cadherin and p120-catenin exhibited normal plasma membrane localization at the tumor centers but were reduced or de-localized at the tumor boundaries. Further, MMP2 was also up-regulated at these same tumor boundaries (**Figure 2**). These results support the view that local cell-cell interactions within the epithelial microenvironment impact tumor invasion and progression and are a potential therapeutic target.

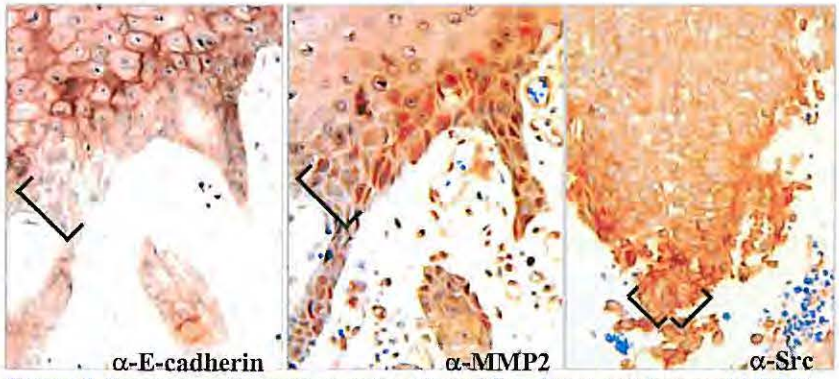


Figure 2. Squamous cell carcinomas show loss of E-cadherin and increased MMP2 and— in some tissues— total Src at tumor edges (*e.g.*, brackets).

<u>*Research progress overview*</u>**:**

The Cagan laboratory developed Csk/Src Drosophila models to explore specific aspects of overgrowth and metastasis. Both Src and the Csk paralog Chk have been implicated in breast cancer metastasis. We used these dCsk/Src metastasis models to identify candidate compounds that reduce the oncogenic-like effects of activated Src. Specifically, we utilized a model that contained 'crumpled wing' and 'overgrown eye' phenotypes due to reducing dCsk activity in the eye and wing (*eye/wing>dCsk*^{RNAi}; Figure). The precise genotype was *GMR-GAL4/FM6, MS1096-GAL4 UAS-dCsk*^{RNAi}*/ MS1096-GAL4 UAS-dCsk*^{RNAi}. We screened the National Cancer Institute "Diversity Set" of 1990 compounds for chemicals that suppressed the *eye/wing>dCsk*^{RNAi} phenotype. The Diversity Set contains an eclectic collection of compounds that emphasize cancer-related compounds. Compounds scored as suppressing the *eye/wing>dCsk*^{RNAi} phenotypes were confirmed in at least two additional re-tests.

356 compounds (18.2%) permitted animal viability but altered the *GMR>dCsk-IR* phenotype. Of these, 251 drugs had an enhancing effect, and 99 compounds (5.0%) had a suppressive effect. 6 drugs had different effects (suppression *vs.* enhancement) at different concentrations. The 99 compounds that initially suppressed the *GMR>dCsk-IR* phenotype were re-tested in multiple wells. 39 compounds demonstrated consistent phenotypic suppression (**Table** below), resulting in a 39.4% repeat rate and 2.0% overall rate of suppressing compounds in the NCI set. This relatively high number of hits likely represents enrichment for cancer-active compounds in the Diversity Set. An example of a hit (Jervine) is provided in the Figure. One compound—2-Phthalimidoglutaric acid— is structurally similar to

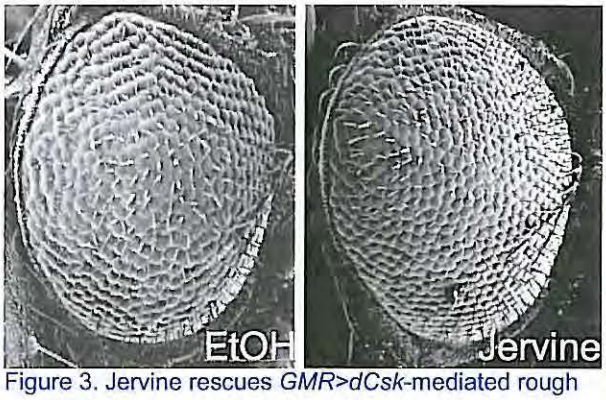


Figure 3. Jervine rescues *GMR>dCsk*-mediated rough

Chemical Name	Known functions (incomplete)
2-Hydroxyethyl-3-hydroxypropyl sulfide	
S-(Carboxymethyl)isothiourea	Potential agonist of GABAA and g - aminobutyric acid . Can inhibit Nitrous Oxide synthesis
Ureidosuccinate, *N*-Carbamoyl-*L*-aspartic acid	An intermediate in pyrimidine biosynthesis and, as such, a regulator of cellular metabolism
(4-*tert*-Butyl-2-chlorophenoxy)acetic acid	
2-(2-Chlorobenzyl)succinic acid	Active in a screen against IMP-1 metallo-b-lactamase.
S-Benzoylcysteine	
O-Ethyloxime-octamethylcyclopentanone	
β-Hydroxyhistidine	
Phenacylthioglycolic acid	Efficacy in mouse CD2F1 leukemia model
N-2-Naphthyl-acetoacetamide	Active in suppressing growth in NCI cancer cell line screen
N,N-Dimethyl-*N'*-2-pyridylsulfamide	
2,2-Diphenyl-3-methyl-4-dimethylamino)butyronitrile	Active in suppressing growth in NCI cancer cell line screen
β-Oxo-α-[(2-pyridinylamino)methylene]-cyclopropanepropanenitrile	
Sodium 4-ethoxybenzenediazosul-fonate	
2-Phthalimidoglutaric acid	Thalidomide analog; affects blood vessel density. Some activity in multiple mouse tumor models and on tumor and endothelial cell proliferation.
2-[[(3,4-Dichlorophenyl)methyl]thio]-4,5-dihydro-1H-imidazole	Active in suppressing growth in NCI cancer cell line screen (simple thioureas known to be toxic)
β, β '-dibromo-*N*-methyl-diallylamine	
CAS# [957362-90-2]; NSC 120571	
2-(methylamino)-*N*-[(4-methylphenyl)sulfonyl]-ethanimidoyl chloride	Can regulate metabolism. Active in suppressing growth in NCI cancer cell line screen; imidoyl chloride expected to be reactive
4,4'-(1,2-ethanediyldi-4,1-piperidinediyl)bis[7-chloroquinazoline]	Anti-obesity activity.
Bis(2-amino-4-sulfonamidophenyl)disulfide	Antimicrobial agents. Active in suppressing growth in NCI cancer cell line screen and CDF1 leukemia mouse.

Chemical Name	Known functions (incomplete)
Mitomycin derivative T-53, Aziridinomitosene	DNA crosslinker; effective as an anti-cancer agent; active in suppressing growth in NCI cancer cell line screen and leukemia models
α-[1-(4-Pyridylmethylene)inden-3-yl]-4-pyridinemethanol	Active in suppressing growth in NCI cancer cell line screen
2-(4-Chlorophenyl)-5-methyl-7-(4-methyl-1-piperazinyl)-[1,2,4]triazolo[1,5-α]pyrimidine	Smooth muscle cell growth inhibitors. Active in suppressing growth in NCI cancer cell line screen
3-[(1-Methyl-4-nitro-5-imidazolyl) thio]-5-(3-pyridinyl)-1,2,4-triazole	Active in suppressing growth in NCI cancer cell line screen; known pro-drug functionality releases another thiourea.
N-phenyl-*N'*-(2,2,6,6-tetramethyl-4-piperidinyl)-1,4-benzenediamine	Inhibits Ornithine decarboxylase activity in tumor cells; active in suppressing growth in NCI cancer cell line screen
Mequitazine, LM 209, Virginan, 10-(3-Quinuclidinylmethyl)phenothiazine	Antihistimine; active in suppressing growth in NCI cancer cell line screen; active in mouse CDF1 leukemia models
2-methyl-a-[2-(1-naphthalenylmethyl)phenyl]-benzenemethanimine	Active in suppressing growth in NCI cancer cell line screen
CAS # [903637-76-3]	
Flavazine, Acid Yellow L	
6-(*p*-chlorobenzylthio)-9-(tetrahydropyran-2-yl)-9H-purine	Active in suppressing growth in NCI cancer cell line screen
Biocan, Ampyrox, Restropin	
6-Chloro-7-sulfamoyl-1,1-dioxide-4H-1,2,4-benzothiadiazine-3-propionic acid	Nitric oxide enhancing diuretic compound
2-(1-(4-(2-pyridyl)piperazino))naphthazarin	Identified as toxic in yeast cell cycle mutant strains. Active in suppressing growth in NCI cancer cell line screen.
Jervine	Inhibitor of Hedghog pathway effector Smoothened. Active in suppressing growth in NCI cancer cell line screen.
Mixture of 4,5,6-trichloro-2-methoxy-pyrimidine and 2,4,5-trichloro-6-methoxy-pyrimidine	Active in suppressing growth in NCI cancer cell line screen
2-[(2-Benzothiazolylthio)acetyl]-3-hydroxy-thiazolo[2,3-*b*]benzothiazolium	Can increase cell proliferation at high doses.
(*S*)-4-ethyl-3,4,12,14-tetrahydro-3,14-dioxo-1H-pyrano [3',4':6,7] indolizino[1,2-b]quinolin-4-yl glycine ester monohydrochloride	Pro-drug derivative of camptothecin. Active in suppressing growth in NCI cancer cell line screen and CDF1 leukemia mouse.
Aphidicolin glycinate	DNA polymerase inhibitor; antitumor activity. Active in suppressing growth in NCI cancer cell line screen.

Table . 39 NCI Diversity Set compounds suppressed the *dCsk-IR* phenotype.

Thalidomide, and we determined that Thalidomide itself also suppressed the *dCsk-IR* phenotypes as well as proliferation in a mouse breast cancer model (see below).

Jervine is a well-characterized steroidal alkaloid and inhibitor of Hedgehog pathway signaling. Jervine is chemically related to Cyclopamine, and both act through suppression of the Hedgehog receptor Smoothened. The Hedgehog pathway has been recently linked to cancer and, recently, metastasis and has generated significant interest by pharmaceutical companies as a potential cancer target. Jervine consistently though mildly suppressed the *dCsk* phenotype in the eye (Figure above) and wing (not shown). The Hh pathway chemical inhibitor AY9944 also suppressed the *dCsk* phenotype whereas Tomatidine, a Jervine/Cyclopamine-related compound with no Hh activity, did not (Vidal *et al*, *submitted*; **Figure 3**).

Importantly, genetic mutations that reduced Hedgehog pathway activity led to suppression of *dCsk*-mediated 'metastasis' in our wing and eye models; conversely, mutations in the pathway inhibitor *patched* enhanced migration of cells (not shown). Interestingly, reducing *dCsk* activity in the wing led to expanded expression of the Hh pathway reporter ptc>GFP including within migrating cells, while reducing Hh pathway through *smo* mutations or through chemical inhibition of Hh signaling suppressed MMP activity (**Figure 4**; Vidal *et al*, *submitted*). Further, reducing Hh pathway activity in the eye mildly but consistently suppressed the *GMR>dCsk-IR* phenotype, while over-expression of the Hh pathway effector Ci enhanced (not shown). These genetic data provide further support for the model that Hedgehog signaling plays an important role in Csk/Src-mediated

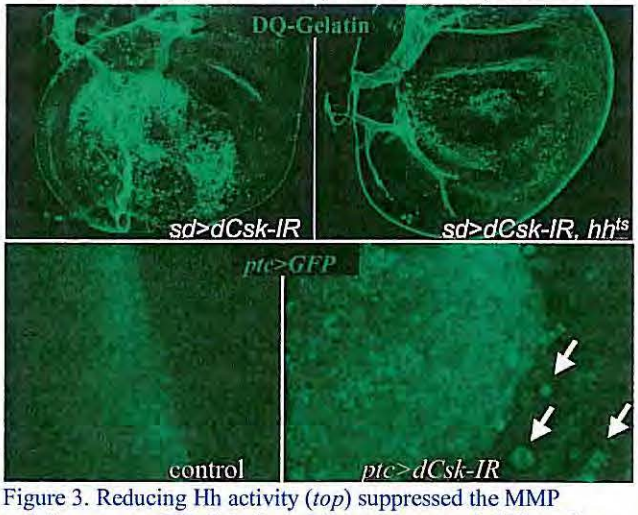


Figure 3. Reducing Hh activity (*top*) suppressed the MMP activity reporter DQ-Gelatin and (*bottom*) expanded Hh pathway activity.

metastasis. I am not proposing to follow through on the role of *Drosophila* Hedgehog because this is an excellent beginning project for Marcos Vidal as he starts his own laboratory.

Drosophila genetics point to functional link between Src and Hedgehog in tumors: The ability of Hh inhibitors to reduce tumor-like phenotypes in both fly and mouse suggested a link between high Src activity and Hh pathway activation. Our preliminary data in developing *Drosophila* wing discs support this view: for example, in $dCsk^{RNAi}$ 'tumors' Hh pathway activity increased as assessed with the pathway reporter *ptc-lacZ* .

Hedgehog inhibitor from Drosophila screen validated as effective anti-tumor compound in murine model of breast cancer: Working with the compounds identified in the *Drosophila* compound screening, the **Cagan** and **Weilbaecher** laboratories collaborated to demonstrate that jervine and cyclopamine suppressed expression of the Hedgehog target *Gli-1* in both 4T1 and B16 murine tumor cells (not shown) and reduced proliferation of fluorescently labeled 4T1 breast cancer cells in cell culture (**Figure 5**; Hirabayashi et al, *in review*). In a syngeneic breast cancer model, daily injection of 50 mg/kg of cyclopamine consistently prevented invasion of 4T1-*GFP-FL* cells (10^5 cells injected into the left ventricle of Balb-C mice) to the lung and bone. By day 9, tumor mass was reduced an average of more than four-fold (**Figure 5**). Cyclopamine reduced MMP2 expression in the lung metastases and decreased tumor induced weight loss/cachexia suggesting an overall survival advantage. As further validation, we demonstrated that 5/6 other compounds that were identified from the *Drosophila* screen showed a similar reduction in viability (**Figure 6**).

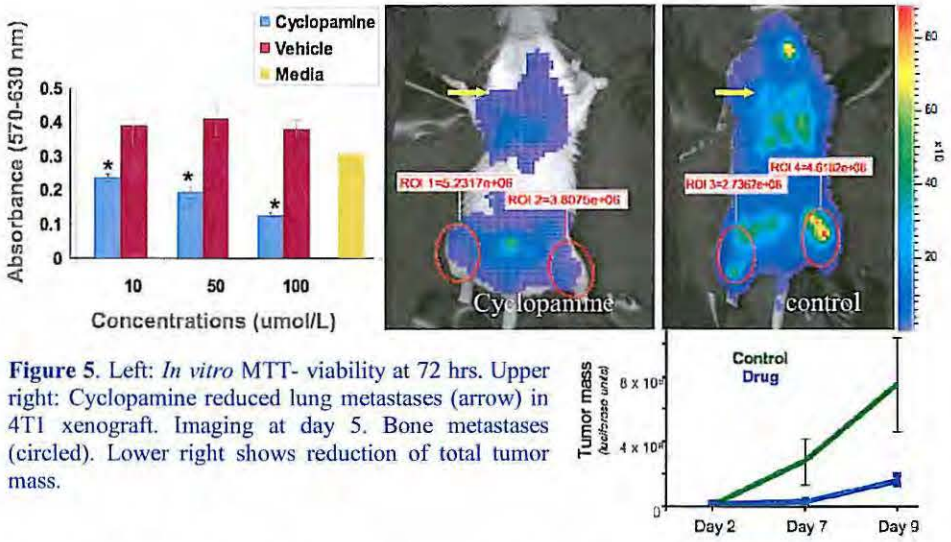


Figure 5. Left: *In vitro* MTT- viability at 72 hrs. Upper right: Cyclopamine reduced lung metastases (arrow) in 4T1 xenograft. Imaging at day 5. Bone metastases (circled). Lower right shows reduction of total tumor mass.

KEY RESEARCH ACCOMPLISHMENTS:

1) Identification of 6 compounds that decrease metastasis in Drosophila metastasis model and decrease viability of mammalian breast cancer cells in vitro.
2) Validation of the compound Cyclopamine, a hedgehog inhibitor, to block lung and bone metastases in murine breast cancer xenograft.
3) This work provides a template for moving candidate therapeutic compounds from fly to mouse.
4) The Drosophila and mouse data demonstrate an important connection between Src and Hedgehog signaling in mediating metastasis.
5) Clinical trial written to evaluate Hedgehog inhibitor GDC-0449 in patients with locally advanced breast cancer (see **Appendix 1** for **CTEP letter of intent).**

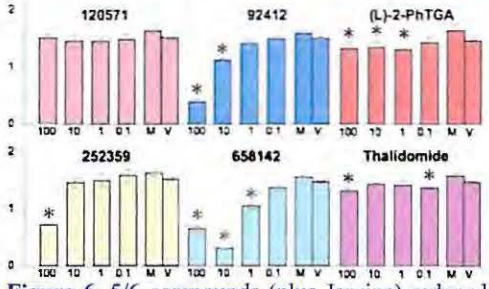


Figure 6. 5/6 compounds (plus Jervine) reduced proliferation after 72 hrs incubation. X-axis: [μM]; Y axis: arbitrary luciferase units.

REPORTABLE OUTCOMES:

This work was presented at the International Cancer and Bone society meeting in Edinburough, Scotland in July 2008. Emanuela Heller, Marcos Vidal, Jill Fink, Lorena Salavaggione, Lourdes Ylagan, Mark Watson, Mark Wilkins, Ross Cagan and Katherine

Weilbaecher. Fly to mouse: a new approach to cancer metastasis. *Cancer Treatment Reviews*, 2008, Supplement.

CONCLUSIONS:

We propose to combine our expertise to target a process that is critical to metastasis that is likely conserved in flies, mice and humans. The advantages of addressing the question of metastasis through the combined expertise of the Cagan and Weilbaecher labs is that we will use the powerful genetic tools provided by *Drosophila* that will identify key genetic pathways critical to tumor cell migration and metastasis that can be rapidly and rigorously tested. This a real time, in vivo dynamic screen that occurs in a whole organism. Tumors develop in the epithelial layer of the wing and the genetics of tumor cell invasion and migration throughout the organism can be modeled in real time, and genetically manipulated in large scale genetic screens. Dr. Weilbaecher's laboratory will take advantage of the genetic knowledge gained from the *Drosophila* metastasis models in the development of an improved breast cancer metastasis mouse model. Dr. Cagan's laboratory will be provided with mammalian human and murine breast cancers to validate their genetic and pharmacologic anti-metastasis strategies. Jointly, Drs. Cagan and Weilbaecher propose to develop novel therapeutics targeted to the metastatic process. In year one, we have identified 6 compounds that decrease metastasis in Drosophila metastasis model and decrease viability of mammalian breast cancer cells in vitro. We have validated the compound Cyclopamine, a hedgehog inhibitor, to block lung metastases in murine breast cancer xenograft and will use this as a template for testing other candidate therapeutic compounds from fly to mouse. We have uncovered a previously unknown and important connection between Src and Hedgehog signaling in mediating metastasis. Finally, we have written a clinical trial using the NCI/CTEP hedgehog inhibitor, GDC-0449, to inhibit microscopic bone marrow metastases in women with locally advanced breast cancer.

REFERENCES:
None

APPENDICES:
CTEP letter of intent

	PHASE I, II, or I/II LETTER OF INTENT Submission Form v2.0	
	National Cancer Institute Division of Cancer Treatment and Diagnosis Cancer Therapy Evaluation Program	

To complete the form electronically, use the mouse pointer or the Tab key to navigate. Select and enter text for each text field. To easily see text fields, go to **Tools|Options** *from Word's menu, click the* **View** *tab, and in the* **Show** *block select '***Always***' from the* **Field Shading** *drop down list.*

Lead Group/Institution:	Washington University School of Medicine
Lead Institution/Group CTEP ID:[1]	MO011
Other Institutions/Groups on study:	
Title of LOI:	A phase II trial of GDC-0449 in women with locally advanced breast cancer
Agent(s) supplied by NCI:[1]	GDC-0449
Commercial Agents in Study:	None
Tumor Type: *(Click within the [[ ]] and type 'x' to indicate the tumor type)*	[[ ]] Solid Tumor [[ ]] Hematologic Malignancy (NOS) [X] Disease-Specific (breast cancer)
Disease-Specific:[1] *(Specify the Name and Code of the Study Disease)*	1. Invasive Breast Carcinoma (10006190)
Performance Status:	ECOG 0, 1
Abnormal Organ Function Permitted?	No
Prior Therapy:	
Phase of Study:	II
Eligibility Criteria:	**Inclusion Criteria:** 1. Women with a histologically confirmed newly diagnosed clinical stage II and III invasive breast cancer; 2. Eligible for standard neoadjuvant chemotherapy; • Regimens containing trastuzumab for HER2 positive disease are allowed 3. ECOG Performance score 0-1;

[1] Detailed Institution, Group, Agent NSC, and Disease codes are available on the CTEP Home Page at http://ctep.cancer.gov/guidelines/values.html

4. At least 18 years of age;

5. The following laboratory values obtained ≤14 days prior to registration: Absolute neutrophil count [ANC] ≥1500/μL; Platelet count ≥100,000/μL; Serum creatinine ≤1.5x upper limit of normal [ULN], Bilirubin ≤1.5 x ULN, and AST and ALT levels ≤3x ULN;

6. Capable of understanding the investigational nature, potential risks and benefits of the study and able to provide written informed consent;

7. Negative serum pregnancy test ≤ 7 days of registration for women of childbearing potential;

Exclusion criteria:

1. Known distant metastasis;

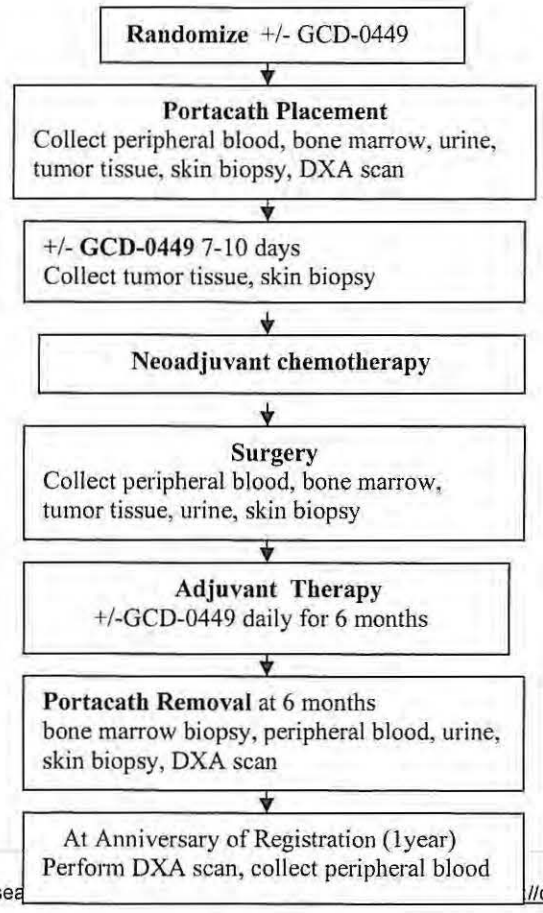
2. Any concurrent chemotherapy, radiation therapy, or use of other any investigational agents;

3. History of other malignancy ≤5 years with the exception of basal cell or squamous cell carcinoma of the skin, treated with local resection only, or carcinoma in situ of the cervix;

4. Patients with an active severe infection; known infection with HIV, hepatitis B virus, or hepatitis C virus;

5. Pregnant, breastfeeding, or of childbearing potential without using dual forms of effective contraception.

Treatment Plan: **Schema**



[1] Detailed Institution, Group, Agent NSC, and Disea //ctep.cancer.gov/guidelines/values.html

Patients are randomized to either arm A (GCD-0449) or B (control).

Randomization: After entry into the trial, patients will be randomized to either arm A or B. Randomization will be performed manually by assigning sealed envelopes to each entered patient containing the enclosed treatment arm. A list of randomized assignments will be generated in advance using a formal probability model implemented by SAS proc plan (v.9.1.3 or higher). Envelopes will be held by the project manager in a locked file cabinet. Each envelope will be entirely opaque, numbered to preserve the order of assignments and opened only at the time of randomization. Informed consent will be obtained from each patient prior to randomization.

Experimental drug therapy:

Arm A:

- Window of opportunity biomarker study: patients will receive GCD-0449 150mg once daily orally for 7-10 days prior to initiation of neoadjuvant chemotherapy. A research biopsy of the primary tumor and skin will be performed at baseline and within 24hours after the last dose of GCD-0449.

  Based on PK data from phase I studies of GDC-0449, we anticipate the achievement of steady state drug concentration after 7-10 days therapy for PD marker evaluation.

- Adjuvant evaluation: following recovery from definitive breast cancer surgery, patients will again receive GDC-0449 150mg PO once daily orally for 6 months or a shorter duration if unacceptable toxicities develop, or if there is withdrawal of consent or by physician decision.

  The dosing of GCD-0449 for arm A is based on the phase I data (as discussed in the background section) demonstrating that 150mg PO once daily iachieves an efficacious steady state drug concentration which is comparable to higher doses, and associated with effective target inhibition and clinical efficacy [1,2]. Importantly, this dosing was found to be well tolerated with even prolonged therapy[2]. In patients with basal cell carcinoma, the median duration on study was176+ (ranges from 39 days to 438+) days[2]. The adjuvant treatment duration of 6 months in our study was chosen arbitrarily and is based on prior experience of GDC-0449 as described above as well as safety and efficacy considerations inferred from adjuvant studies of other agents in breast cancer. For example, a duration of 9-week of trastuzumab in combination with chemotherapy was able to achieve a cure in a large number of patients in the FinHer trial, although 1 year was chosen for most studies. In the case of chemotherapy, usually 2-6 months of therapy is required for the maximum benefit.

Arm B (control): Patients will be treated with standard neoadjuvant chemotherapy, surgery and adjuvant therapy without GCD-0449.

Neoadjuvant and adjuvant therapy (Arms A and B):

Patients will receive standard neoadjuvant and adjuvant therapy (including adjuvant endocrine, trastuzumab and radiation therapy (as recommended by the treating physicians). Patients may start a bisphosphonate following the DXA bone density test performed at 6 months of adjuvant GDC-0449 (arm A) or control therapy (arm B).

Portacath placement and baseline tissue sampling (Arms A and B):

Portacaths will be placed for central venous access to administer medications such as chemotherapy and for blood sampling before starting therapy in all patients under

[1] Detailed Institution, Group, Agent NSC, and Disease codes are available on the CTEP Home Page at http://ctep.cancer.gov/guidelines/values.html

sedation in the operating room.

Baseline research bone marrow, peripheral blood, skin, and tumor specimens will be collected for patients at the time of Portacath placement to minimize patient discomfort.

Portacath removal and 6 month tissue sampling (Arms A and B):

Six month research bone marrow, peripheral blood, skin, and urine specimens will be collected for patients at the time of Portacath removal to minimize patient discomfort.

Research biopsy of the primary tumor:

Arm A: 2 core biopsy specimens (1 fixed in 10% formalin and 1 fresh frozen) of the primary tumor will be collected at baseline (prior to GCD-0449, during portacath placement), following 7-10 days of GCD-0449 (prior to the initiation of neoadjuvant chemotherapy) and at the time of breast surgery.

Arm B: 2 core biopsy specimens (1 fixed in 10% formalin and 1 fresh frozen) will be collected at baseline (at the time of portacath placement) and at the time of breast surgery.

Research bone marrow biopsy (Arms A and B):

A total of 20 milliliters of bone marrow will be aspirated from 2 sites (left and right iliac crests) in EDTA containing tubes at baseline (at the time of portacath placement), breast cancer surgery and 6 months following adjuvant administration of GDC-0449 (arm A) or control (arm B). Samples will be further processed in Drs Aft and Watson's laboratories for analysis of disseminated tumor cells.

Research blood collection (Arms A and B):

Research blood will be collected at baseline (during portacath placement), the time of surgery, following 6 months of experimental (or control) therapy and then every 6 months for 5 years. 20 milliliters of blood will be collected in EDTA containing tubes for serum and plasma. In addition, 10 milliliters of blood will be collected for circulating tumor cells.

Research skin biopsy (Arms A):

2 skin punch biopsy specimens will be collected prior to (at the time of portacath placement) and after 7-10 days of neoadjuvant administration of GDC-0449. Skin biopsy will be repeated at the time of surgery and at 6 months following adjuvant administration of GDC-0449.

Research DXA bone mineral density (Arms A and B):

We will assess the BMD post surgery prior to adjuvant GDC-0449 (arm A) or control (arm B) and following 6 months of adjuvant treatment and at the 1 year anniversary of enrollment

Rationale/Hypothesis:

Overview: Evidence from the literature and our preliminary data suggests that signaling pathways involved in stem cell renewal and epithelial to mesenchymal transition (EMT) may contribute to TN tumor pathogenesis and disease progression. In addition, micrometastases that persist despite chemotherapy are likely enriched with cells that have stem cell like features, leading to subsequent disease recurrence. In recent literature, hedgehog (Hh) signaling has been shown to be important for the maintenance of breast cancer stem cells [3-5]. Strikingly, cyclopamine, a Hh inhibitor, was identified during a high throughput screening for compounds able to inhibit tumor growth and metastases by our co-investigator Dr. Kathy Weilbaecher and her collaborators. Further study demonstrated that cyclopamine inhibited

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cancer cell viability and tumor metastasis in breast cancer models. This pre-clinical data supports further clinical investigations of Hh inhibitors in breast cancer. The safety profile of the Hh inhibitor GDC-0449 with prolonged therapy in phase I studies justify its evaluation in patients with high risk early stage disease.

We hypothesize that the Hh inhibitor GDC-0449 can reduce residual micrometastatic disease following chemotherapy in patients with locally advanced breast cancer. Patients with clinical stage II/III breast cancer with a plan for neoadjuvant chemotherapy would be eligible for this randomized phase II study of GDC-0449 versus no GDC-0449 in the adjuvant setting. The primary endpoint is to evaluate bone marrow for the presence of micrometastases, also called disseminated tumor cells (DTCs), and their expression levels of putative stem cell markers as well as genes involved in the Hh signaling and the EMT process before and following adjuvant GDC-0449 therapy. To analyze GDC-0049 induced molecular changes in the primary tumor, patients in the experimental arm will also receive 7-10 days of GDC-0449 for biomarker analysis (window of opportunity study). In addition, the effect of GDC-0449 on bone turn over and bone mineral density will be assessed.

1. Hedgehog signaling, and breast cancer stem cell

Adult stem cells are slow-dividing and long-living population of cells that have the capacity for self-renewal and multilineage differentiation and comprise a small proportion of the total tumor cell population. The cancer stem cell theory is based on the identification of $CD44^+/CD24^-/Lin^-$ human tumor cells which form tumors in a mouse xenograft with as few as 100 cells, while tens of thousands of tumor cells of other phenotypes are unable to do so [6]. The existence of cancer stem cells is thought to explain the failure of chemotherapy and other treatments to eradicate metastatic disease. In a recent publication by Li et al, chemotherapy treatment increased the percentage of $CD44^+/CD24^-$ cells and increased mammosphere formation efficiency [7].

Hh signaling (Fig.1.) has shown to be important for the maintenance of breast cancer stem cells[3,4]. Using *in vitro* culturing and a xenograft mouse model, Liu et al examined the role of Hh signaling and Bmi-1 in the maintenance of normal and malignant human mammary stem cells[4]. They demonstrated that Hh target genes, including PTCH1, GLI1 and GLI2, were highly expressed in these cells and were down-regulated when the stem cells were allowed to differentiate. Additionally, stem cell renewal was increased following treatment with Sonic Hedgehog (Shh) but decreased following treatment with cyclopamine, a Hh inhibitor.

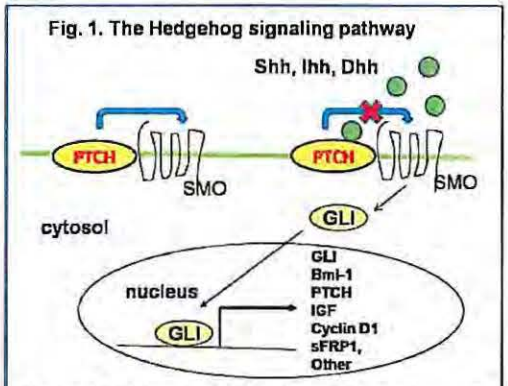

Fig. 1. The Hedgehog signaling pathway

Binding of Hh ligands to PTCH removes its repression on SMO, which activates the GLI family transcription factors.

Enhanced GLI-mediated transcription by overexpression of GLI2 in breast epithelial stem or progenitor cells resulted in ductal hyperplasia when placed in the mammary fat pad of immunocompromised mice [4]. This observation suggests that increased Hh signaling in breast epithelial stem cells may contribute to the development of epithelial precursors of cancer[3]. Breast cancer stem cells, characterized by the cell surface phenotype $CD44^+CD24^{-/low}$ and tumor initiating capacity, were found to express PTCH1, GLI1 and GLI2 at higher levels than other cancer cells, suggesting activation of the Hh pathway in these cells [4,5]. Therefore, inhibition of Hh activity in cancer stem cells may decrease or prevent breast cancer recurrence. From this data, we propose that a Hh inhibitor which could target the cancer stem cell renewal process would decrease the risk of recurrence.

2. The Hedgehog (Hh) signaling pathway and cancer

The Hh signaling pathway (Fig.1) is important in epithelial-mesenchymal interactions and

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cell differentiation during embryonic development [8]. The cancer promoting effects of Hh signaling are achieved by enhancing cell growth, inducing epithelial to mesenchymal transition (EMT), mediating stromal-epithelial interactions, and regulating stem cell self-renewal[9]. Inactivating mutations of *PTCH1*, activating mutations of *SMO (Smoothened)*, and loss or mutation of SUFU leads to basal cell carcinomas, medulloblastomas and rhabdomyosarcomas. However, overexpression of the Hh ligand, often Shh, and the Hh transcriptional targets such as GLI1, is believed to activate Hh signaling and may be important in the development and progression of a variety of cancers, including cancers of the esophagus, stomach, pancreas, liver, lung, prostate, ovary, and breast[9,10].

Stromal Hh signaling has also been shown to be critical for tumorigenesis in Hh-expressing cancers [11]. Antagonizing Hh signaling in tumor stroma, by deleting *SMO* in the stroma or using small molecule inhibitors of Hh, inhibited tumor growth in xenograft models[11], arguing for a paracrine mechanism of Hh signaling. Therefore, inhibition of Hh signaling not only likely to have anti-tumor effects in tumors that are intrinsically dependent on the Hh pathway, but also in tumors that rely on activated stromal Hh signaling. Interestingly, overexpression of Hh ligands has been observed in a number of epithelial tumor malignancies including colorectal, endometrial, ovarian, and pancreatic cancers, in which a paracrine Hh signaling is proposed to be important for tumor growth[11].

3. Hh and breast cancer

The importance of Hh signaling in breast carcinogenesis and cancer progression is just beginning to be elucidated[9]. Unlike in basal cell carcinoma, mutations in Hh signaling components are uncommon. Although in an initial preliminary tumor screening, a somatic H133Y mutation of Shh was identified in 1 of 6 breast cancers[12] and a mutation in *PTCH1* was identified in 2 of the 7 breast cancers analyzed[13], a subsequent larger study of 84 primary breast carcinomas did not detect this H133Y of Shh. No mutations were found in the *PTCH1* coding region in 45 primary breast tumors and no mutation was identified for smoothened (*SMO*) in 48 samples[14]. Missense mutations in GLI1 were identified in 2/24 (8%) breast cancers and 1/11 (9%) breast cancer cell lines [15]. Loss of the chromosomal region containing *PTCH1* was found in 19% of primary breast cancers and 33% of breast cancer cell lines by array based comparative genomic hybridization [16]. A functional role for any of these identified mutations in breast cancer has not been proven. However, a biallelic Pro1315Leu (C3944T) polymorphism of *PTCH1* was significantly associated with breast cancer. This polymorphism modified an association between oral contraceptive use and breast cancer risk in pre-menopausal women[17].

In a recent publication, methylation of the *PTCH1* promoter was found in a pharmacological-based global screen for epigenetically silenced tumor suppressor genes in MCF-7 and MDA-MB-231 breast cancer cells[18] and correlated with low PTCH1 expression[18]. Low PTCH1 expression was found to be associated with higher tumor grade and lower ER expression in the study. Interestingly, GLI1 expression was identified in tumors with high or low PTCH1. These data indicate both ligand-dependent and -independent activation of the Hh pathway in breast cancer [18].

Several studies have reported overexpression of Hh ligands and Hh target genes in breast cancer. The overexpression of Shh ligand and GLI1, a target of Hh signaling, in a subset of breast cancers suggests that activation of the pathway occurs by ligand overexpression. In a series of 52 human breast carcinomas, high intensity for GLI1 and over-expression of Shh when compared with adjacent normal tissue were detected in 100% of the specimens by IHC [19,20]. By using qRT-PCR, the mean levels of PTCH1 and GLI1 were also shown to be modestly increased (less than 2-fold) in about 40% of the breast cancers compared to normal breast in an analysis of 15 breast cancer and 6 normal breast[10]. Similarly, GLI1 mRNA and/or protein were 2- to 17-fold higher in the microdissected epithelium from 5 of the 10 (50%) breast cancers and Shh expression was elevated in 63% of the breast cancers in comparison to normal breast tissues from the same individual[21]. The expression of PTCH1 is variable, possibly due to promoter methylation[9].

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Hh pathway activation is also implicated in the aggressiveness of the breast cancer. In an analysis of 121 invasive ductal carcinoma samples, GLI2, but not GLI1, overexpression was statistically correlated with negative PR expression, high Ki-67 proliferating index, node metastasis, and higher clinical stage of breast cancer [22]. This finding suggests that GLI2 was active as a key GLI family gene in the mammary gland, and played a more pivotal role than GLI1. This is consistent with the hypothesis that Hh signaling molecules play an important role in the progression of invasive ductal carcinoma of breast.

Mukherjee et al reported that Hh signaling differs in epithelial and stromal cells in benign breast and breast cancer, indicating increased Hh signaling in a subset of breast cancer in both stroma and epithelial components [21]. Using microdissected tissues from frozen samples and qRT-PCR and IHC, it was demonstrated that GLI1 mRNA and protein, along with PTCH1 and Shh proteins, were increased by 1.8 to 18-fold in epithelial cells of 3 of 10 breast cancers[21]. In addition, GLI1 mRNA and/or protein were higher in cancer-associated stroma than normal stroma in these 3 cancers[21]. GLI1 mRNA was higher in fibroblasts than in the epithelial cells both in cell lines and tissue studies and the expressions of SMO and GLI2 were higher in the breast fibroblasts, supporting a paracrine mechanism[21].

The biologic significance of Hh signaling activation in breast cancer has been evaluated in several studies in the literature. In mouse models with mutations of ptch1 or constitutively activated Smo are not predisposed to mammary cancer development[9]. However, mice heterozygous for targeted disruption of ptch1 have ductal hyperplasia and dysplasias of the mammary gland[23]. Interestingly, disruption of ptch1 in mammary stroma was required for the development of the ductal changes. In addition, MMTV driven activated Smo resulted in ductal dysplasia in transgenic mice[24]. These suggest that Hh signaling is sufficient to induce ductal hyperplasia but additional molecular abnormalities are required for tumorigenesis[9]. Kubo et al examined a panel of breast cancer cell lines including BT-474, SK-BR-3, MDA-MB231, MCF-7, for the signaling activity and growth dependence on Hh signaling. All of the 4 cell lines showed increased expressions of Shh, PTCH11 and GLI1, with nuclear staining of GLI1 identified in all except MDA-MB231. Cyclopamine, a steroidal alkaloid that blocks the Hh pathway, but not tomatidine (an inactive analogue of cyclopamine), suppressed the proliferation of three breast carcinoma cell lines, except MCF-7, in a dose- and time-dependent manner and accompanied by decreased GLI1 and GLI1 mediated transcription [19,20]. Similarly, in the study reported by Mukherjee et al, cyclopamine reduced the viability of breast cancer epithelial cell lines, accompanied by decreased GLI1 and GLI1 mediated transcription, but not the fibroblasts or epithelial cells from benign breast [21]. In addition, an association between estrogen receptor alpha and hedgehog pathway was suggested in breast cancer in a recent publication[25].

In addition, Hh signaling has also been implicated in tumor-induced osteolysis in breast cancer xenograft models[26]. GLI2 was found to be expressed in several cancer cell lines that cause osteolytic lesions *in vivo* and produce parathyroid hormone related peptide (PTHrP), a major factor involved in tumor-induced osteolysis in breast cancer, but not in the nonosteolytic and non PTHrP secreting cancer cell lines. Stable expression of GLI2 resulted in increased production of PTHrP in vitro and enhanced osteolysis in vivo, suggesting that osteolysis in human breast cancer is driven at least in part by Gli2[26]. Recent data indicates that Runx2, a key factor for bone formation, directly up-regulates Indian Hedgehog (IHH) by binding to its promoter to activate Hh signaling in cancer cells.

Furthermore, Hh signaling has shown to be important for the maintenance of breast cancer stem cells as discussed in the earlier section [3 4].

These studies provide a biological rationale for evaluating Hh signaling as a therapeutic target for breast cancer[9,19,20]. There are still many questions remaining regarding the role and the mechanisms of Hh signaling in breast cancer tumorigenesis and disease progression. In the current study, we propose to investigate whether an Hh inhibitor can reduce cancer recurrence in high risk triple negative breast cancers, with an emphasis on

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correlative studies to investigate the potential effects of Hh inactivation on the expression of EMT and putative stem cell markers on bone marrow micrometastases from patients enrolled in the study.

4. GDC-0449

a. Drug information

GDC-0449 is a small molecule antagonist of the Hh signaling pathway identified by a small molecule compound library screening. GDC-0449 is over 100-fold more potent and has more favorable pharmacologic properties than cyclopamine. GDC-0449 blocks Hh-stimulated signaling *in vitro* with an IC50 of 13nM (in murine 10T1/2) and 2.8nM (in human HEPM). Its selectivity was confirmed *in vitro* by luciferase reporter assays. Dramatic anti-tumor activity was observed in preclinical xenograft models of tumors that are dependent on Hh pathway such as basal cell carcinoma and medulloblastoma. In the D1523 colorectal cancer xenograft model, the effect of GDC-0449 on Hh activity was mainly observed in the stroma rather than the tumor epithelium by GLI1 expression although anti-tumor effect was observed in this model[27,28]. Further study on a panel of colorectal cancer xenograft models indicated that the anti-tumor efficacy correlates with Hh ligand expression[28], supporting the paracrine mechanism of Hh signaling in tumorigenesis.

Pharmacokinetics (PK) and pharmacodynamic (PD) studies have been conducted extensively in preclinical models. Based on the available preclinical information and the 4.6-fold greater potency on human Hh compared to the mouse, a minimum trough level of at least 3-5 uM may be required to target human stroma[27]. The preclinical toxicology studies indicated a reversible dose-dependent decrease in body weight, accompanied by reduced food consumption, in rats and dogs following oral administration of GDC-0449[27]. In addition, spermatogenesis was affected. In addition, there is concern of skeletal side effects from Hh inhibitors [29]. No mutagenic or clastogenic effects have been observed for GDC-0449[27].

b. Clinical experience

The results of a phase I study of GDC-0449 administered at daily doses of 150mg, 270mg and 540mg (single dosing on day 1, then continuous dosing starting day 8 for PK purpose) have been reported [1,2]. There were no dose-limiting toxicities. Cumulative drug related toxicities, including dysgeusia (or altered taste sensation) (15.8%), fatigue (10.5%), and asymptomatic hyponatremia (10.5%), were relatively mild (mostly grade 1) and occurred after prolonged exposure. Reversible drug-related asymptomatic Grade 3 hyponatremia and fatigue (1 each) were reported with prolonged drug administration[1]. Partial responses were observed in 6 of the 9 patients with refractory basal cell carcinoma at doses of 150mg and 270mg, with a median duration on study of 176+ (ranges from 39 days to 438+) days[2]. Similar steady state high plasma drug concentrations (30-35 μM, which is well above the predicted efficacious concentration for stroma Hh signaling inhibition) were achieved after 1 week of continuous daily dosing at all dose levels. The terminal half life was determined to be 10-14 days in healthy volunteers. GLI1 was down modulated >2-fold in skin biopsies most patients analyzed. Based on PK, PD data, continuous oral dosing at 150 mg/day was recommended for phase II studies [1]. Phase II clinical trials of GDC-0449 in colorectal, ovarian and basal cell carcinomas are ongoing.

5. Preliminary data

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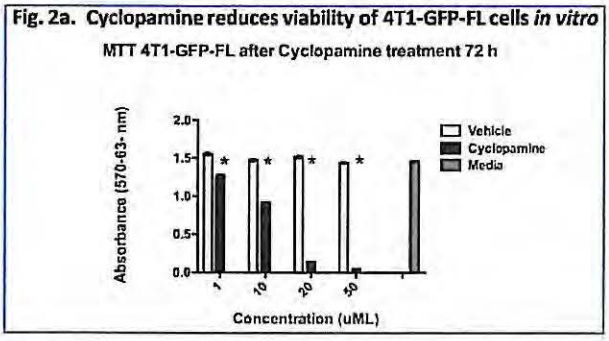
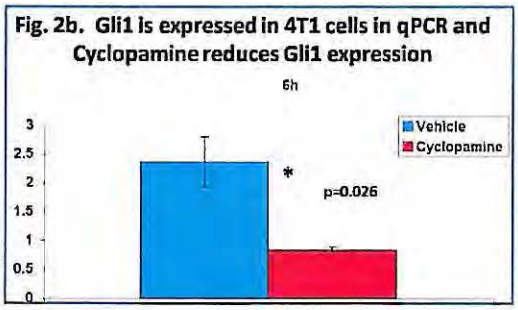
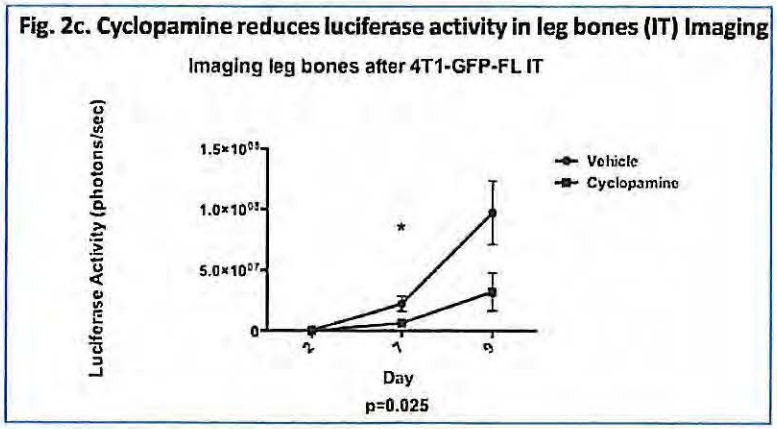
1) **Preclinical study indicating Hh inhibitors decreases breast cancer cell viability *in vitro* and metastasis *in vivo*.** This work was performed by our co-investigator Dr. Kathy Weilbaecher and her collaborator Ross Cagan. Briefly, Jervine and cyclopamine, Hh inhibitors, were identified in their high throughput screening for compounds with anti-tumor and anti-metastasis properties in a novel Drosophila whole animal compound screening model. Not only did Jervine/cyclopamine exert potent inhibitory effects on tumor growth and metastasis in *Drosophila*, but also reduced breast cancer cell viability *in vitro* (Fig.2a) and metastasis *in vivo* (Fig.2c) by using the murine 4T1 triple negative breast cancer cell model. The decrease in cell viability was associated with a reduction in Hh signaling as assessed by GLI 1 mRNA expression (Fig.2b). Cyclopamine administration led to a decrease in breast cancer metastatic tumor burden in lungs and leg bones after intra-arterial injection of 4T1-GFP-FL cells (green fluorescent protein and firefly luciferase labeled 4T1 cells) as assessed by bioluminenscence imaging on days 2, 7 and 9 after tumor injection (Fig.2c). This data indicates that Hh inhibitors could be effective therapeutic agents for breast cancer.







2) **Analysis of bone marrow micrometastatic cells (DTC) (data from Drs. Aft and Watson)** In patients who did not achieve pCR, about 50% were found to have bone

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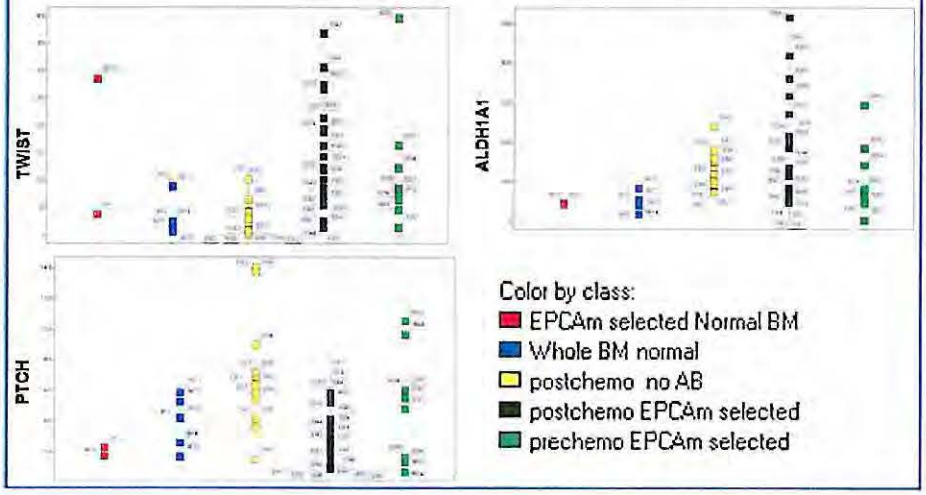
marrow micrometastases in the study described above. Effective immunoenrichment of DTCs using the EpCAM antigen and gene expression analysis of the DTCs from patients' bone marrow samples have been successfully established in the laboratory of the study co-PI, Dr. Aft and co-investigator Dr. Mark Watson[30]. Using bone marrow samples from 25 breast cancer patients (enrolled in the study described above) as well as 2 healthy volunteers, only selection using the antibody against EpCAM, but not antibodies to *ABCG2, CD44, CXCR4, BSG* (EMMPRIN), *SPP1* (osteopontin), and *CASP3* (Sca-1) antigens achieved enrichment of the DTCs[30]. Unsupervised hierarchical cluster analysis on the EpCAM selected bone marrows showed distinct gene expression profiles. Importantly, postchemotherapy EpCAM selected bone marrow samples are enriched with transcripts of genes implicated in cancer growth, metastasis, including TWIST [30]. Subsequent qRT-PCR confirmed that the expression of TWIST predicted early recurrence [30].

Analysis of the gene array data, demonstrates that ALDH1 expression is over represented in post-neoadjuvant chemotherapy bone marrow samples enriched by EpCAM (Fig.3). Interestingly, several samples showed elevated PTCH1 expression in non-selected samples, which could suggest an up-regulation of Hh signaling in the bone marrow stroma since the amount of EpCAM positive cells are small.

Fig.3. qRT-PCR of TWIST, ALDH1A1 and PTCH in bone marrow aspirates from patients treated with neoadjuvant chemotherapy



4) **Tumors enriched with Hh signaling are at a high risk of relapse (data from Drs. Aft and Watson)**. As part of a correlative science companion study to Dr. Aft's neoadjuvant chemotherapy trial described above, we previously performed gene expression profiling on tumor biopsies, pre- and post-neoadjuvant therapy. To evaluate the potential relationship between Hh signaling and tumor relapse, we re-examined the expression profile of several gene transcripts associated with Hh signaling in this cohort of both ER+ and "triple negative" breast tumors, before and after neoadjuvant chemotherapy. Fig.4. shows unsupervised clustering of all tumor samples, based on the expression of multiple probe sets for Hh signaling genes including *SHH, IHH, GLI2, GLI3,* and, *PTCH.*

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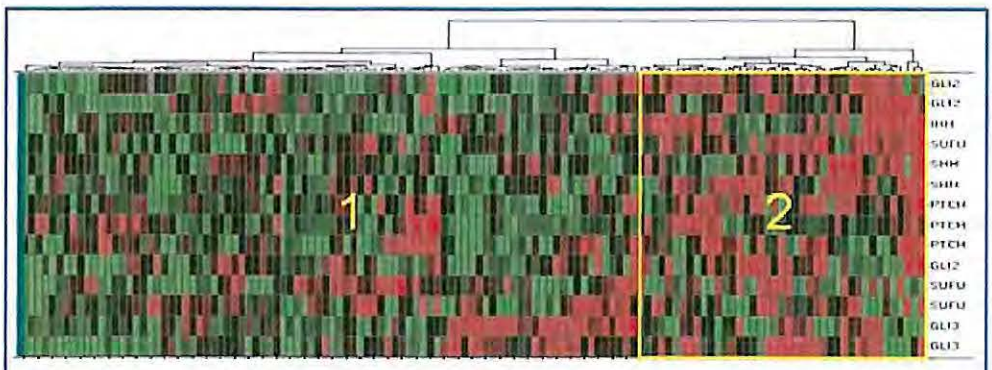

Fig. 4. Unsupervised clustering of 128 ER+ and triple negative breast tumors, both before and after neoadjuvant therapy, based on Hh signaling gene expression.

When considered as a group, tumors segregated into two distinct classes: those with high Hh associated gene expression (Cluster 2) and those with generally lower Hh associated gene expression (Cluster 1). Among pre-therapy tumor specimens, 11 / 27 (40%) of high-Hh class tumors were ER+ whereas 16 / 27 (60%) of "triple negative" tumors were of the high-Hh expression class. More importantly, when considering only post-therapy (i.e. incomplete pathological response) triple negative tumors, the subject population of this proposal, we found that only 4 / 10 (40%) cases in the low Hh expression class demonstrated early distant recurrence, whereas 4 / 5 (80%) cases in the high Hh expression class had early distant metastasis. Although this is only a small, retrospective analysis, it suggests that activated Hh signaling (measured using a whole tumor gene expression surrogate) may correlate with early relapse, specifically in patients with triple negative breast cancer.

In summary, Evidence suggests that signaling pathways involved in stem cell renewal and the EMT may contribute to breast cancer pathogenesis and disease progression. We hypothesize that the Hh inhibitor GDC-0449 will target the Hh signaling in the micrometastatic cancer cells that have cancer stem cell properties. Our preclinical data further supports the evaluation of Hh inhibitors as therapeutic agents in breast cancer.

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Laboratory Correlates:

1. Existing FFPE or frozen tumor blocks from prior biopsy or breast surgery will be collected for the following studies: (both arms A and B)
    a. To further define the breast cancer molecular subtyping by PAM50 qRT-PCR or IHC
    b. To analyze baseline Hh signaling pathway, including ligands (Shh, Ihh, Dhh), and direct targets (GLI1, GLI2,PTCH1, PTCH2, HHIP, Blimp1, sFRP1 and GLI 3) by qRT-PCR and IHC.
    c. To analyze for stem cell markers and EMT markers such as CD44,

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ALDH1, SNAIL, TWIST etc

2. Serial tumor biopsy of the primary breast cancer at baseline during portacath placement and following 7-10 days of drug therapy (Arm A)
    a. To analyze Hh signaling changes by qRT-PCR and IHC of GLI1 expression
    b. To analyze changes in the levels of MMP2, stem cell and EMT markers
    c. To analyze changes in tumor cell apoptosis (by Tunnel assay or Cleaved caspase 3) and proliferation (by Ki67)
3. Serial skin punch biopsies at baseline during portacath placement and following GDC-0449 (at 7-10 days following neoadjuvant GDC-0449 and again following 6 months of adjuvant GDC-0449) (Arm A)
    a. To analyze the expression of GLI 1 expression for Hh signaling inhibition by IHC
4. Serial research blood collection (baseline during portacath placement, at the time of breast surgery, 3 and 6 months following adjuvant GDC-0449 or control therapy and every 6 months during the follow up) (Arms A and B)
    a. To analyze bone turnover markers (including serum bone specific alkaline phosphatase, osteocalcin, and N-telopeptide)
    b. Circulating tumor cells for the expression of ALDH1, etc
5. Bone marrow collection (baseline during portacath placement, at the time of surgery, and following 3 and 6 months of adjuvant GDC-0449 or control therapy) (both arms A and B):
    a. To look for the presence of DTC by both cytokeratin staining and qPCR for TWIST
    b. To analyze EpCAM selected (tumor cell) and non-selected (stroma) for Hh signaling pathway genes as discussed above and the expression of TWIST, ALDH1 etc
6. Bone mineral density test (post surgery, and following 6 months and 1 year of adjuvant therapy) for therapy effects on bone density

Endpoints/Statistical Considerations:

The Primary Objectives:

1. Effect of 6 months of GDC-0449 on the clearance of bone marrow micrometastasis
2. Effect of 6 months of GDC-0449 on bone turn over markers and bone mineral densities
3. Effect of neoadjuvant GDC-0449 on molecular markers in the Hedgehog signaling, apoptosis and proliferation in tumor and skin tissues

The Secondary Objectives:

1. Toxicity profile with neoadjuvant and adjuvant administration of GDC-0449
2. DFS and OS with adjuvant GDC-0449

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Exploratory Objective:

1. To correlate neoadjuvant GDC-0449 induced biomarker changes in the primary tumor or skin with effects of adjuvant GDC-0449 on micrometastasis, bone marker turn over, bone mineral density and DFS.

Study Design

Randomized phase II design: Patients will be randomly assigned to receive standard therapy or standard therapy with GDC-0449.

Sample Size and Power Consideration: Generalized estimating equations will be used to estimate the odds of DTC in bone marrow at baseline 6 months. The expected proportion with DTCs in bone marrow at the time of surgery is 35% if negative at baseline and 41% if positive at baseline. The proposed sample will provide at least 80% power to detect a difference in the proportion of patients with DTC in bone marrow in arm A (with GDC-0449 treatment), relative to the proportion positive in arm B (standard therapy) if treatment with GDC-0449 reduces the 6 month frequencies to ~10% in patients positive and those negative at baseline. Bone mineral density and bone turnover markers are approximately Gaussian and can be analyzed with mixed linear repeated measures models. Given values of bone mineral density observed at baseline and 6 months, and correlations of ~ 0.9 between values at these two time points, in the control group of the recently completed clinical trial, the proposed sample will provide at least 80% power to detect a difference of 33% at the hip (-0.18 in arm B vs. -0.12 in arm A), 13% at the lumbar spine (-0.39 in arm B vs. -0.45 in arm A) and 27% at the wrist (-0.22 in arm B vs. -0.16 in arm A). Data from the same trial indicate that baseline to 3 month correlation in bone turnover markers is ~50%. The proposed sample will provide at least 80% power to detect a difference of 62% in N-Tx (13.0 in arm B vs. 8.0 in arm A) and 45% in osteocalcin (3.5 in arm B vs. 1.6 in arm A). A 9-fold difference in bone alkaline phosphatase will also be detectable with 80% power (0.09 in arm B vs. -0.8 in arm A). Preliminary data on molecular markers indicative of Hedgehog signaling, apoptosis and proliferation in human tumor and skin tissues is not currently available, so no power calculation has been attempted.

Toxicity: As per NCI CTC Version 3.0, the term toxicity is defined as adverse events that are classified as either unrelated, unlikely to be related, possibly, probably, or definitely related to the study treatment. The maximum grade for each type of toxicity will be recorded for each patient, and frequency tables will be reviewed to determine toxicity patterns. In addition, we will review all adverse event data that is graded as 3, 4, or 5 and classified as either "unrelated or unlikely to be related" to study treatment in the event of an actual relationship developing.

Relapse-free survival: Relapse-free survival time is defined as the time from registration to documentation of disease progression (using the RECIST criteria), second primary disease or death without documentation of disease relapase. The distribution of progression-free times will be estimated using the method of Kaplan-Meier.

Overall survival time is defined as the time from registration to death due to any cause. The distribution of survival times will be estimated using the method of Kaplan-Meier.

Estimated Monthly Accrual: 40 patients a year

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Proposed Sample Size:	120
Earliest date the study can begin:	January 2010
Projected Accrual Dates: *(Month/Year format)*	Start: January 2010 End: December 2012
To document accrual rate, list trials with patients who had similar Tumor Type/Phase of Study/Prior Therapy:	.
Protocol Number / Title / Sponsor:	Wash U HSC#02-0788 / Impact of Neoadjuvant Chemotherapy with or without Zometa on Occult Micrometastases and Bone Density in Women with Locally Advanced Breast Cancer / Siteman Cancer Center
Trial Activation / Trial Completion Dates:	*(Include NCI Number if NCI-sponsored)* March 2003 / March 2006
No. of Patients Enrolled:*	120 (similar eligibility criteria) *(*Entire study or to date, if study is not completed. Only include patients enrolled at site(s) relevant to LOI proposal)*
List all Active, Approved, or In Review studies at your institution for which this patient population will be eligible:	**No competing trials.**
Protocol Number / Title / Sponsor:	
Trial Activation Date / Anticipated Completion Date:	
No. of Patients Enrolled to Date / Patient Enrollment Period / Duration of Patient Enrollment / Total planned Patient Enrollment:*	
Is this LOI part of an NIH Grant, Cooperative Agreement or Contract?	
If yes, provide the Award Number:	
Will this study receive support from non-NCI sources (i.e., industry, ACS)?	N
If the proposed trial includes correlative studies, CTEP	

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assumes funding is available to support them.	
If yes, is it Grant funding?	Pending
If yes, provide the Grant Number:	
Is this a Career Development LOI?	N Further information and instructions regarding the submission of a Career Development LOI may be found at http://ctep.cancer.gov/documents/loi_instructions.html
If yes, please attach and check off the following:	PI curriculum vitae [ ] Institutional letter of commitment [ ] Mentor letter of commitment [ ]

The **Investigational Drug Steering Committee** (IDSC) is designed to provide NCI with broad external scientific and clinical input for the design and prioritization of phase I and phase II trials with agents for which CTEP holds an IND. Membership of the IDSC includes the Principal Investigators of phase I U01 grants and phase II N01 contracts, representatives from the NCI Cooperative Groups, NCI staff members, and additional representatives with expertise in biostatistics, correlative science technologies, radiation oncology, etc., as well as patient advocates and community oncologists, as needed. Experts with specific expertise will be included as ad hoc members for consideration of specific agents. The current membership list may be found at: http://ccct.nci.nih.gov/steering-committees/idsc

Periodically the IDSC will assess LOIs from a strategic perspective to determine whether the Clinical Development

Plan for an agent should be modified. When requested by CTEP, the IDSC will provide input on LOIs to assist in CTEP decision-making.

For unsolicited LOIs only: Please check one of the following options *(Note: While selecting an option is required, neither choice will affect the outcome of the CTEP review of this LOI)*:

This LOI may ☐ /may not ☐ be looked at by the IDSC.

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Group Chair/Cooperative Agreement-PI (GCCA-PI) Name:	**Charles Erlichman, MD**
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Non Group Grant-PI Phone:	[Click and enter Phone No.]
Non Group Grant-PI Fax:	[Click and enter Fax No.]
Non Group Grant-PI E-mail:	[Click and enter E-mail Address]

Please submit Letter of Intent forms (LOIs) to the Protocol and Information Office (PIO) via e-mail at:

pio@ctep.nci.nih.gov, Attention: LOI Coordinator

Notes: LOIs from Cooperative Group must be submitted through the Group Operations.

Proposals for trials to be conducted under a Cooperative Agreement must include complete contact information for the Principal Investigator and Protocol Chair.

Questions? Please call LOI Coordinator at (301) 496-1367.

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